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Xenopus ElrB, but not ElrA, binds RNA as an oligomer: Possible role of the linker

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Abstract **We have shown that ElrA and ElrB, *Xenopus* ELAV homologues, bind the Vg1 mRNA 3′UTR translation element in *Xenopus* oocytes and implicated ElrB in mediating translational repression during oogenesis. Here we report that, while ElrA and ElrB are 69% identical and both exhibit RNA binding in the nM range, recombinant ElrB, but not ElrA, is able to oligomerise. This oligomerisation is also seen with the endogenous protein. Both RNA binding and oligomerisation require the linker region flanked with two RNA recognition motifs. Our data demonstrate a novel and unique property of ElrB which may be important for its function as a translational regulator.**

Keywords: ELAV proteins; Oligomerisation; *Xenopus* oogenesis; RNA binding

1. Introduction

The ELAV-like proteins are RNA-binding proteins named after the first member, ELAV (embryonic lethal abnormal vision), identified in *Drosophila* [1]. In vertebrates, the ELAV-like family contains four members, ElrA, ElrB, ElrC and ElrD, produced from different genes [2]. In humans, the homologues are named HuR, Hel-N1 (HuB), HuC and HuD. All the ELAV proteins have a characteristic structure of three RNA recognition motifs (RRMs) with RRM1 and RRM2 in tandem followed by a linker region and a terminal RRM [3–6]. Although the RRMs are conserved amongst the ELAV family, the N-terminus and the linker region are somewhat diverse [7].

Previous studies have shown that ELAV proteins interact specifically with AU-rich elements (AREs) in the 3′ untranslated regions (UTRs) of various mRNAs [4,8–11] and they have been implicated in key mechanisms of post-transcriptional regulation including mRNA stability, translation and alternative splicing [12–15]. A more recent study shows that the translational repression of CAT-1 mRNA, which is mediated by the micro RNA miR-122, can be relieved by HuR [16].

In *Xenopus* oocytes, only two ELAV proteins are expressed, ElrA and ElrB, and both are found in the cytoplasm [3,17]. We have previously shown that, during oogenesis, these two proteins specifically bind the Vg1 translation element (VTE). Vg1 encodes a protein involved in mesoderm induction and the mRNA is both localised and translationally repressed during oogenesis. The VTE is a 250 nt, AU-rich element, in the 3′UTR of the Vg1 mRNA, which represses the translation of a reporter RNA, both in vivo and in vitro, to the same extent as the full length 3′UTR [11]. The expression pattern of ElrB during early oogenesis is most consistent with a role for ElrB, rather than ElrA, in the translational control of Vg1 mRNA [17]. Disruption of the consensus ElrB binding site in the VTE abolished the repression of the reporter RNA while multiple copies of this same binding site supported translational repression. Most importantly, injection of antibody against ElrA and ElrB resulted in the synthesis of Vg1 protein [17].

In this study, we show that recombinant ElrB, but not ElrA, oligomerises, and that endogenous ElrB oligomerises during the early stages of oogenesis when Vg1 mRNA translation is repressed [17]. We also determine that the oligomerisation of ElrB is RNA-dependent and requires the linker region flanked by two RRMs. The data suggest a specific ability of ElrB to oligomerise that may be functionally relevant in vivo.

2. Materials and methods

2.1. Cloning of recombinant proteins

Constructs containing the *Xenopus* ElrA and ElrB coding regions were kindly provided by Peter Good (Accession Nos. U17596.1 and U17597.1, respectively). The coding sequences for the full-length proteins and the subdomains were amplified by PCR. The primers were designed to introduce an *Nco*I site at the 5′-end and an *Xho*I site at the 3′-end. These sites were used to insert the PCR products into the vector pGIT [18] to allow the expression of C-terminally His-tagged proteins. The forward primer for ElrA was 5′-TGCCATGGGTAACGGTTATGAAGATCAC-3′, the reverse primer was 5′-TTGCGGCCGCCCTCGAGTTTGTGTGACTT-3′. The forward primer for ElrB, B1, B12 and B12L was 5′-TGCCATGGCAGTCAGACTGTGTGAT-3′; B2 and B2L3 was 5′-TGCCATGGGTTCGGCATCAATAAGGGAT-3′; B3 was 5′-TGCCATGGGACATGCTGGTACAGGATGG-3′. The reverse primer for ElrB, B2L3, and B3 was 5′-TTGCGGCCGCCCTCGAGAGCTTTGTGTGTTTT-3′; B12L and B2L was 5′-TTGCGGCCGCCCTCGAGAAAGTTAATCCCAGCCAAAC-3′; B12 and B2 was 5′-TTGCGGCCGCCCTCGAGATTGTTGGCAAACTTCACCG-3′; B1 was 5′-TTGCGGCCGCCCTCGAGTCTGGCATAGGATACTTTAA-3′.

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Abbreviations: VTE, Vg1 translation element; VLE, Vg1 localisation element; RRM, RNA recognition motif; ARE, AU-rich element; UTR, untranslated region; rRNA, ribosomal RNA; K_d, dissociation constant; RNP, ribonucleoprotein

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2.2. Expression and purification of His-tagged recombinant proteins in Escherichia coli

Chemically-competent BL21 star (DE3) cells (Novagen) were electroporated with the appropriate plasmids. The cells were grown in 2×TY at 37 °C to an A_{600} of 0.4 and induced with 1 mM IPTG for 3 h at 37 °C. Following centrifugation, the cell pellets were resuspended in 25 ml HNTA (1 M NaCl, 50 mM sodium phosphate buffer, pH 7.8, 1% Triton X-100) supplemented with 2× Complete Protease Inhibitors (Roche), 2 mM benzamidine and 3.5 mM β-mercaptoethanol and the cells lysed using a French press. Following centrifugation, the soluble fractions were mixed with 1 ml of HNTA-equilibrated Ni-NTA resin (Qiagen) and incubated at 4 °C with rotation for a minimum of 1 h. The beads were washed consecutively with HNTA, PBS and PBS supplemented with 50 mM imidazole and then the recombinant proteins were eluted off the Ni-NTA beads into 1 ml of PBS containing 1 M imidazole and passed through a PD10 column (Amersham) to exchange the buffer to protein buffer (150 mM NaCl, 50 mM sodium phosphate buffer, pH 7.8, 1 mM DTT, 15% glycerol, 0.1% Triton X-100). The concentrations of the His-tagged proteins were determined by Bradford assay and by SDS–PAGE analysis of protein dilutions against BSA standards.

2.3. Preparation of oocyte lysates

Mixed stage lysate was prepared as described in [11] while staged lysate was prepared according to [19].

2.4. RNA synthesis

To generate a uniformly radiolabelled probe for filter binding and band shift assays, 0.5 μg of linearised pVLE [17] and pVTE [11] were used as a template for transcription by T7 RNA polymerase in the presence of [α^{32}P]-UTP as described [20]. All in vitro transcribed mRNAs were purified by centrifugation through ProbeQuant G-50 Micro Columns (Amersham Biosciences) and quantitated after precipitation with CTAB.

2.5. Band shift assay

Binding reactions containing 100 mM KCl, 1 mg/ml heparin, 0.1 mg/ml competitor *E. coli* ribosomal RNA (rRNA), 2 μl *Xenopus* extract and 10 fmol [^{32}P]-labelled RNA in 1× Binding Buffer (10 mM HEPES, pH 7.2, 3 mM $MgCl_2$, 5% glycerol, 1 mM DTT) were incubated at room temperature for 15 min. To detect a supershift, the reactions were incubated for a further 5 min in the presence of protein A–Sepharose purified antibody. After the addition of 2 μl of 30% glycerol, samples were separated on a 5% non-denaturing gel and exposed to Fuji Super RX X-ray film overnight.

2.6. Filter binding assay

The procedure was adapted from [21]. Reactions containing varying concentrations of His-tagged recombinant protein (1–500 nM) and 1–5 nM [^{32}P]-labelled probe in 1× Filter Binding Buffer (100 mM KCl, 50 mM Tris, pH 8.0, 1 mM $MgCl_2$) were incubated for 10 min at room temperature. The samples were then filtered through a Hybond-C membrane (Amersham Biosciences) using a dot blot vacuum apparatus. Following three washes with Filter Binding Buffer, the membrane-bound fraction of the probe was quantitated using a PhosphorImager screen (Molecular Dynamics).

2.7. Ni-NTA Pull-down assay

Ten μl Ni-NTA beads were pre-incubated with 30 pmol His-tagged recombinant protein in a final volume of 200 μl Pull-down Buffer (100 mM KCl, 1 mM $MgCl_2$, 50 mM HEPES, pH 7.5, 5 mM imidazole, 0.2% NP-40, 5% glycerol) for 1 h at 4 °C. After washing the beads, 10 μl [^{35}S]-labelled protein, obtained by coupled transcription/translation [22], were added and incubated for 2 h at 4 °C. Following washing, the bead-bound fractions were eluted with 30 μl 2× PSB and resolved by SDS–PAGE.

3. Results

3.1. ElrB–VTE complexes differ from ElrA–VTE complexes in supershift gel retardation assays

The level of expression of the two maternally expressed *Xenopus* ELAV proteins differs during oogenesis. While ElrA increases between stages III and VI, ElrB, on the other hand, is present in high amounts in early oocytes, stages I–III, when Vg1 mRNA is translationally repressed. Vg1 translation is activated from stage IV onwards [11] when the level of ElrB dramatically decreases.

To compare ribonucleoprotein (RNP) complexes formed between the VTE and the two ELAV proteins during oogenesis, we performed gel retardation assays with labelled VTE RNA and an equivalent amount of lysate from stage I or VI oocytes (Fig. 1A). Purified peptide antibodies specifically recognising ElrA and ElrB were added to the staged lysates to detect the association of the VTE with individual proteins. The addition of the anti-ElrA antibody gives a single supershift with both

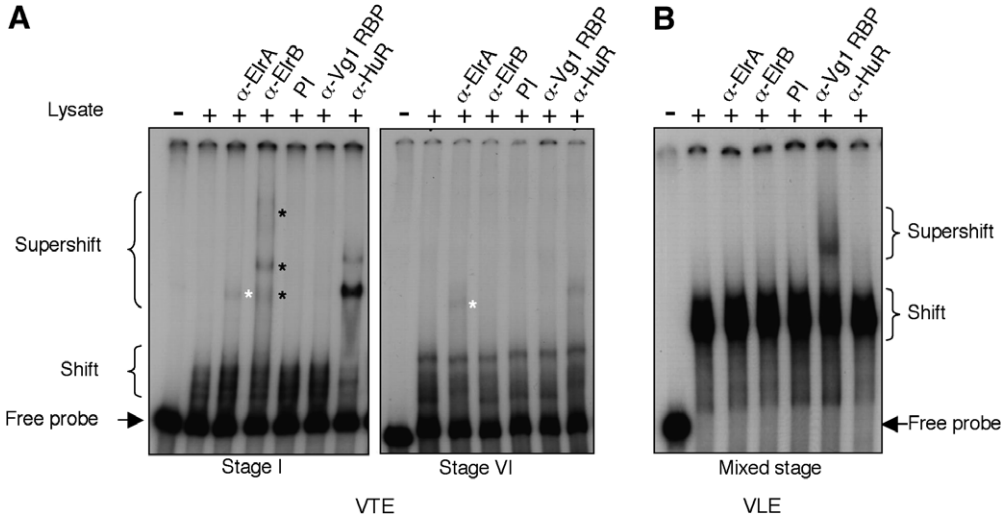

Fig. 1. ElrB-VTE complexes differ from ElrA–VTE complexes in gel retardation assays. (A) Gel retardation assays were performed with one oocyte equivalent of stage I or stage VI lysate mixed with 10 fmol [^{32}P]-labelled VTE probe and the antibodies indicated (2 μl α-ElrA, 5 μl α-ElrB, 3 μl preimmune serum (PI), 3 μl α-Vg1RBP and 0.5 μl α-HuR). The supershift obtained upon the addition of α-ElrA antibody is indicated with a white star and those obtained with α-ElrB antibody are indicated with black stars. (B) One microlitre of *Xenopus* mixed staged lysate (~1 oocyte equivalent) was mixed with 10 fmol [^{32}P]-labelled VLE probe and the indicated antibodies as in (A).

stage I and stage VI lysates. This is consistent with the original UV-cross linking data [11] indicating equal binding of ElrA to the VTE throughout oogenesis. In contrast, the anti-ElrB antibody detects and supershifts a complex in stage I but not in stage VI oocytes, reflecting the decreased level of ElrB in stage VI relative to stage I oocytes [17].

More importantly, we noted that the anti-ElrB antibody results in at least three independent supershifted complexes likely to represent the binding of multiple molecules of ElrB to the VTE probe. In agreement, α-HuR antibody, which essentially detects only ElrB in stage I oocytes, results in at least two supershifted complexes. Preimmune antibody and an antibody raised against Vg1RBP, which binds the Vg1 localisation element (VLE) but not the VTE [11], did not supershift any VTE-containing RNA (Fig. 1A). In contrast, anti-Vg1RBP antibody, but not anti-ElrA or anti-ElrB antibody, induced a shift in a VLE-containing complex (Fig. 1B). These controls validate our hypothesis that endogenous ElrB, unlike ElrA, may be present in multiple complexes when bound to the VTE.

3.2. Both ElrA and ElrB bind RNA efficiently

To investigate the RNA-binding properties of the two *Xenopus* ELAV proteins, recombinant His-tagged versions were purified after overexpression in *E. coli*. The dissociation constants (K_d) of ElrA and ElrB binding to the VTE were determined using a filter-binding assay in the absence of competitors. The K_d of ElrB was calculated to be approximately 3 nM (ranging from 1 to 4 nM) and that of ElrA to be approximately 7 nM (ranging from 5 to 12 nM) (Fig. 2A). Thus, both proteins bind to the RNA with high affinity, with K_ds in the low nM range.

We have previously shown that the VTE possesses AU-rich sequences at the 5′- and 3′-ends that are required for translational repression [11]. Here, poly(A), poly(U) and poly(C) were used as competitors in the filter-binding assay (Fig. 2B). The competitors were added individually to the reactions in 10-, 100- or 1000-fold molar excess over the labelled VTE RNA probe. Only poly(U) completely abolished the binding of both recombinant ElrA and ElrB to the VTE, even at the lowest concentration, suggesting that the recombinant ElrA and ElrB interact mainly with U residues.

The full-length recombinant proteins were also tested in a band shift assay to examine possible differences in complex formation (Fig. 2C). Interestingly, we noted that ElrB but not ElrA, is able to oligomerise, as indicated by the extensive smearing of the probe RNA. Therefore, the recombinant proteins behave in a similar manner to endogenous ElrA and ElrB.

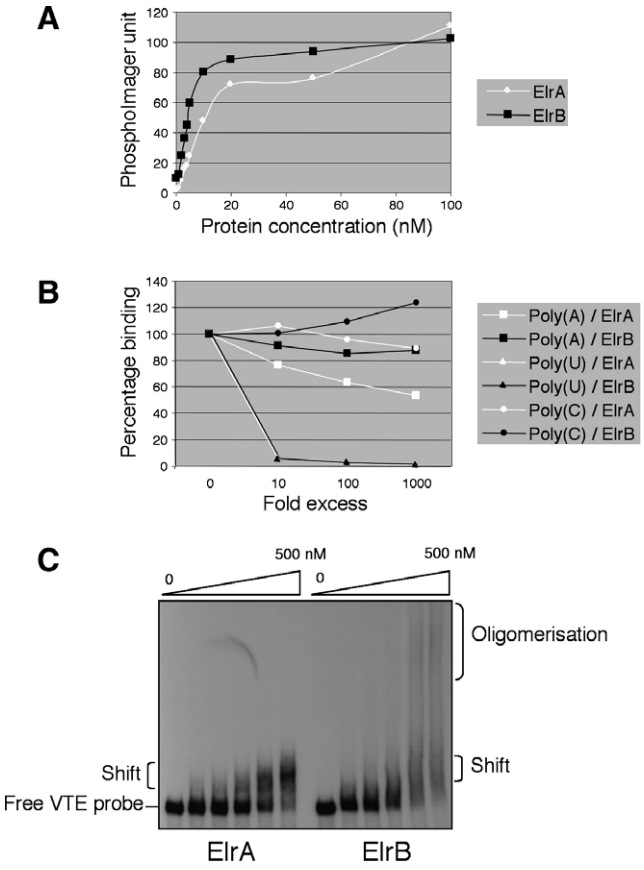

Fig. 2. Both recombinant ElrA and ElrB proteins have high affinity for RNA, but only ElrB oligomerises. (A) Filter binding experiments were performed with 1, 2, 3, 4, 5, 10, 20, 50 and 100 nM ElrA and ElrB bound to 30 fmol [^{32}P]-labelled VTE probe in the absence of competitors. The amount of RNA bound to protein and thus retained on a nitrocellulose membrane was quantitated on a Phosphorimager and plotted against protein concentration. The K_ds for ElrA and ElrB were determined by linear regression using the linear part of the curve (between 1 and 5 nM). (B) Poly(A), poly(U) and poly(C) were used as competitors at 10-, 100- or 1000-fold excess over the labelled VTE probe in filter-binding reactions containing 20 nM recombinant protein. The results are expressed as a percentage relative to the binding in the absence of competitor. The experiment was performed several times and the data shown are representative. (C) An increasing amount (0, 25, 50, 100, 250, 500 nM) of recombinant ElrA and ElrB were incubated with 10 fmol [^{32}P]-labelled VTE and the reaction resolved by native PAGE.

3.3. Determination of the domains responsible for ElrB RNA binding

Different subdomain combinations of ElrB (Fig. 3A) were over-expressed as His-tagged forms in *E. coli*, purified (Fig. 3B) and analysed by filter-binding assay to compare the involvement of each of the RRMs in RNA binding (Fig. 3C). Not all possible combinations of the subdomains were obtainable, due to lack of expression, solubility or purification. The results show that the purified ElrB subdomains can be divided into three categories. The first category, comprising B2 and B3, has a very low affinity for the RNA ($K_d \geqslant 500$ nM). The second category of subdomains (B1 and B12) is able to bind the RNA ($K_d \sim 20$ nM), but they do not bind the RNA as well as the third category, which includes the full-length ElrB and the fragment B2L3 ($K_d \sim 3$ nM).

Next, the ElrB subdomains were examined by band shift assay using the same RNA and recombinant proteins at 25 and 500 nM (Fig. 3D). The full-length protein, ElrB, binds the VTE at a concentration of 25 nM (lane 2) and at 500 nM, when a higher molecular weight shift due to oligomerisation is observed (lane 3). The subdomains B1 and B12 both bind the RNA with distinct shifted complexes seen above the free probe (lanes 6 and 9), albeit with lower affinity than the full-length protein. In contrast, the single RRM fragments B2 and B3 do not shift the RNA probe, even at the highest protein concentration (lanes 12 and 18). The only protein, in the set of subdomains tested, capable of forming a high molecular

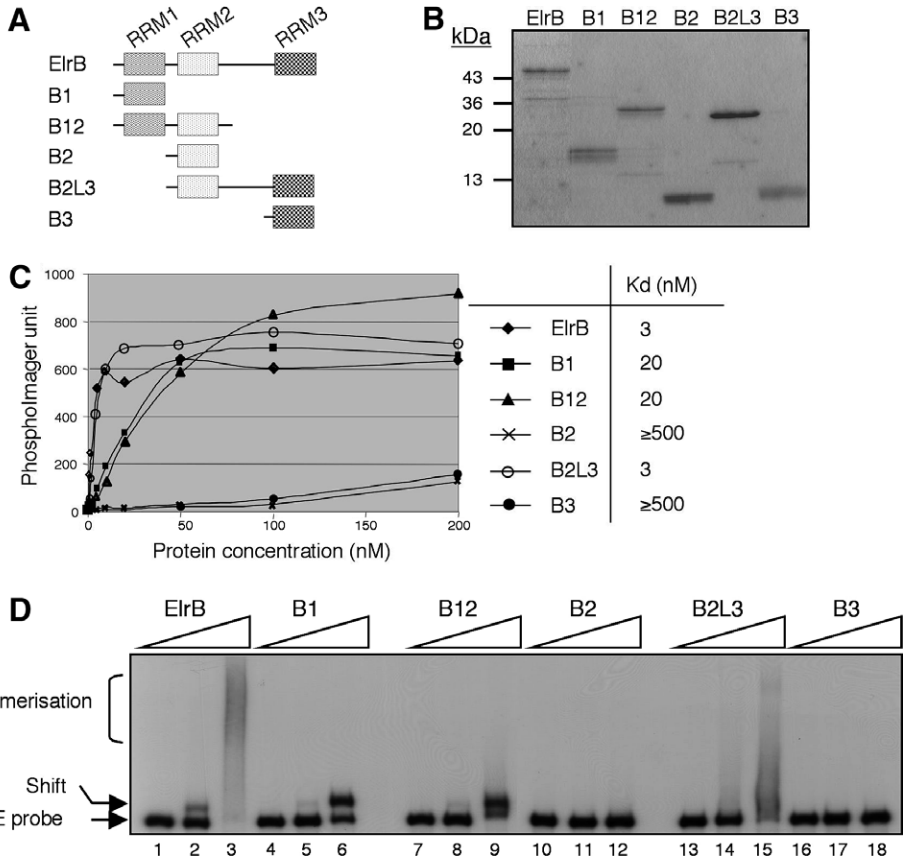

Fig. 3. The roles of individual ElrB subdomains in RNA binding by filter-binding and band shift assays (A) Schematic representation of the purified subdomains of ElrB protein. (B) Coomassie-stained SDS–PAGE of the purified subdomains. (C) Thirty fmoles [^{32}P]-labelled VTE RNA were incubated with an increasing concentration (0, 1, 2, 5, 10, 20, 50, 100 and 200 nM) of full-length recombinant ElrB and each of the purified subdomains (B1, B12, B2, B2L3 and B3), in the absence of competitors and the reactions subjected to filter binding. The experiments were performed in duplicate, then quantitated by PhosphorImager and the resulting values plotted. The K_d values for the full-length ElrB and each of the subdomains were determined and shown in the table on the right. (D) Ten fmoles [^{32}P]-labelled VTE were incubated with an increasing concentration of the recombinant ElrB subdomains (0, 25 and 500 nM) in the presence of 0.1 mg/ml *E. coli* rRNA and analysed by native PAGE and autoradiography. The migration of the free probe and of the shifted complexes are indicated on the left.

weight shift as seen with the full-length protein is B2L3 (lane 15). Therefore, the two proteins that have the highest affinity for RNA, ElrB and B2L3, are also the two proteins that oligomerise, and are the only two proteins in the set of purified ElrB subdomains that contain the linker region. These data suggest that, in the case of ElrB, the linker region may be involved both in RNA binding and in oligomerisation.

3.4. ElrB oligomerisation is RNA-dependent

To examine whether ElrB interacts with itself, and whether such an interaction is direct or requires RNA, we used pull-down assays performed with His-tagged recombinant ElrB bound to Ni-NTA beads and labelled proteins synthesised in rabbit reticulocyte lysate. In order to assay the dependence of oligomerisation on the presence of RNA, the translation reactions were treated with 1 mg/ml RNase A prior to incubation with ElrB-bound Ni-NTA beads (Fig. 4A). Fragments of mRNA, rRNA and tRNA remain after the micrococcal nuclease treatment of the lysate and these could mediate an RNA-dependent interaction. ElrA was used as a control. Labelled ElrA bound weakly and non-specifically to Sepharose and to His-tagged ElrA and ElrB (lanes 4–9). In contrast, a strong interaction between ElrB and itself (lane 17) was detected, as previously, but only in the absence of RNase treatment (lane 18). Therefore, the presence of RNA is necessary for the oligomerisation of ElrB.

3.5. Further study of the ElrB subdomains and the linker region

As the subdomains where the linker region was at the N- or C-terminus could not be purified from *E. coli*, to examine the role of the linker region in oligomerisation, pull-down assays were performed with His-tagged recombinant ElrB and three additional labelled ElrB subdomain variants (B12L, B2L and BL3) synthesised in rabbit reticulocyte lysate.

Fig. 4B shows that only full-length ElrB (lane 4) and the subdomain B2L3 (lane 13) are able to self-associate. Subdomains B12L and B2L are unable to interact with ElrB (lanes 7 and 10) suggesting that the linker alone is not responsible for the oligomerisation of ElrB. BL3 appears to show some interaction with ElrB (lane 17) but not to the same extent as full-length ElrB (lane 4) or B2L3 (lane 13). These data suggest a requirement for the linker region flanked by 2 RRMs for efficient ElrB oligomerisation. In addition to the ElrB subdomains tested, TIA-1 was used as a control. TIA-1 is another AU-rich element-binding protein containing three RRMs that has previously been shown to play a role in the translational repres-

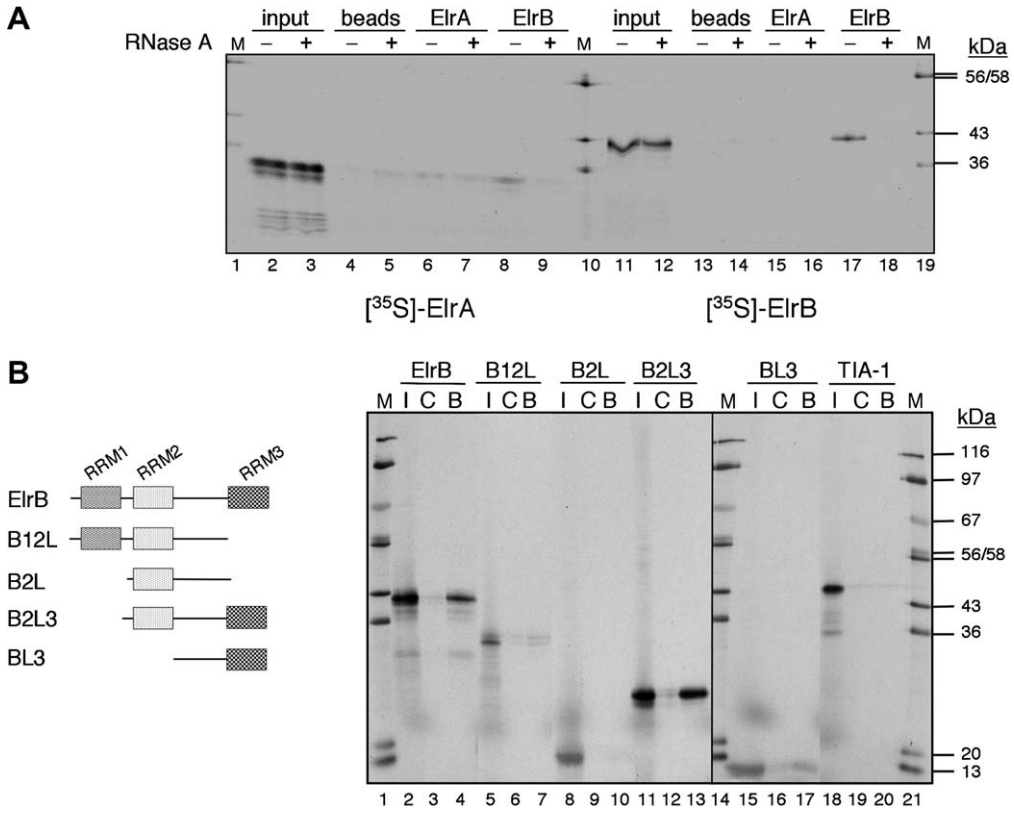

Fig. 4. Self-association of ElrB assessed by pull-down assay. (A) ElrA and ElrB were translated using the coupled transcription/translation system and subjected to a pull-down assay. RNase-treated (+) and non-treated (−) proteins were mixed with 2 μg full-length His-tagged recombinant ElrB or ElrA pre-incubated with 10 μl of Ni-NTA beads or with beads alone. Protein size markers (M) are indicated on the right in kDa. (B) A schematic representation of the subdomains of ElrB protein containing the linker region, obtained using the coupled transcription/translation system is shown on the left. The proteins were translated in the presence of [^{35}S]-methionine resulting in radiolabelled proteins (I) that were subjected to a pull-down assay with Ni-NTA beads alone as a control (C) or with 2 μg of full-length His-tagged recombinant ElrB incubated with 10 μl beads (B). An additional control is shown in the right-hand panel, where His-tagged TIA-1 replaced ElrB. The experiment was performed on three different occasions and the same results seen. Protein size markers (M) are indicated on the right in kDa.

sion of the TNF-α [23] and COX-2 mRNAs [24]. TIA-1 showed no interaction with ElrB (lane 20). These data confirm and extend those obtained in the band shift assays and together suggest that the linker region has to be flanked by both RRM2 and RRM3 for ElrB to bind RNA and to oligomerise.

4. Discussion

The overall domain structure of ELAV proteins is composed of three RRMs, with a linker sequence between RRM2 and RRM3 (reviewed in [25]). The ELAV proteins, in particular the RRM domains, are highly conserved between species and between different members of the family; for example, the human/*Xenopus* orthologs HuR/ElrA and HuB/ElrB are 92% and 93% identical, respectively, and ElrA/ElrB are 69% identical overall. However, only 50% identity is observed in the linker domain between ElrA and ElrB. ElrA/HuR possesses the shortest linker region of 55 amino acids. The key difference between the linker domain in ElrA and ElrB is the insertion of 15 amino acids in ElrB [2,3].

In several studies addressing the question of which portions of ELAV proteins are responsible for RNA binding, it is generally agreed that RRM1 and RRM2 are the principal RNA-binding domains in HuR, HuC and HuD [5,7,26], as well as in ElrA and mHuC [27,8]. The role of RRM3 is less well-defined. RRM3 from HuC and HuD has been shown to bind poly(A) [5,8], while the HuD RRM3 has also been described as enhancing RNA binding by RRM1 [28]. In the case of HelN1/HuB, RRM3 on its own is capable of efficient binding of *c-myc* RNA [4]. These differences may reflect the use of different assays to monitor RNA binding, the examination of different ELAV proteins and RNA targets and the different subsets of domains being compared. Nevertheless, there is broad agreement that stronger binding generally occurs with two RRMs [25]. Our results indicate that, for ElrB with our substrate, highest affinity binding is provided by RRM2-linker-RRM3.

Several investigators have observed that ELAV and ELAV-like proteins are capable of oligomerisation [29,30,14]. In particular, HelN1/HuB was noted to undergo several band-shifts in gel retardation assays [4]. However, no direct functional relevance of the multimerisation has been described. We show here that ElrB, but not ElrA, oligomerises, suggesting a role for the linker domain in oligomerisation. Interestingly, a potential role for the linker in oligomerisation of an ELAV-related RNA-binding protein, EDEN-BP, has been described [29].

Thus, we have shown that ElrB differs significantly from ElrA in its ability to oligomerise. Key proteins, such as FRGY2 and Xp54, which are involved in the formation of translationally repressed mRNPs have also been shown to undergo multimerisation and this is thought to play an important part in their repressive function (reviewed in [31]). Our data, therefore, further support a role for ElrB, rather than ElrA, in the translational repression of the *Xenopus* Vg1 mRNA.

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